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Regulation of Stage-Specific Nuclear Translocation of Dnmt1o during Preimplantation Mouse Development

Adam S. Doherty,*,† Marisa S. Bartolomei,† and Richard M. Schultz*,[1]

**Department of Biology, and †Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

DNA methylation of CpG dinucleotides by DNA methyltransferase 1 is implicated in the regulation of transcription and, in particular, the transcription of imprinted genes. Although the oocyte-specific form of Dnmt1 (Dnmt1o) possesses a functional nuclear localization signal, it is predominantly localized in the cytoplasm of the oocyte and preimplantation mouse embryo but undergoes a transient nuclear localization during the eight-cell stage, when the embryos undergo compaction. We report here that Dnmt1o is likely retained in the cytoplasm by an active process, since ~70% of DNA methyltransferase activity is retained following permeabilization procedures that result in the release of ~75% of oocyte/embryo protein. Treatment of the embryos with agents that disrupt either microfilaments or microtubules has little, if any, effect on the retention of Dnmt1o in permeabilized embryos. While Dnmt1o does not colocalize with either mitochondria or endoplasmic reticulum, it does colocalize with annexin V, which is known to interact with Dnmt1o. We also report that the timing of nuclear entry of Dnmt1o during the eight-cell stage is independent of DNA replication, transcription, and protein synthesis, as well as compaction, cell contact, and cytokinesis. The time of nuclear entry, therefore, appears linked to the time following fertilization, which suggests that a molecular clock governs the time of nuclear import.

Key Words: **DNA methylation; DNA methyltransferase; compaction; preimplantation embryo.**

INTRODUCTION

The faithful methylation of cytosine residues in the CpG dinucleotides of nuclear DNA is crucial to mammalian development (Bestor, 2000; Jones *et al.*, 1998; Nan *et al.*, 1997). While DNA hypermethylation is typically associated with gene repression (Jones *et al.*, 1998; Nan *et al.*, 1997), this modification is postulated to be important for the suppression of retroposon transcription and the regulation of gene expression, particularly that which controls genomic imprinting and X inactivation (Bestor, 2000). Furthermore, perturbation of normal DNA methylation patterns is associated with disease (Jones *et al.*, 1998).

At least four DNA methyltransferase (Dnmt) genes have been identified that encode the *de novo* and maintenance methylase activities that are observed in mammals. The first characterized Dnmt gene, *Dnmt1*, is predominantly responsible for the maintenance of cellular methylation patterns, but it also exhibits *de novo* activity (Yoder *et al.*, 1997). Two additional genes, *Dnmt3a* and *Dnmt3b*, encode methyltransferases with *de novo* activity (Okano *et al.*, 1998). Deletion of these genes in mice results in extensive perturbations in DNA methylation patterns and embryonic lethality (Li *et al.*, 1992; Okano *et al.*, 1999). Likewise, the significance of proper DNA methylation in humans is underscored by the observation that ICF syndrome, which is characterized by immunodeficiency, centromere instability, and facial anomalies, is caused by mutations in the *DNMT3B* gene (Xu *et al.*, 1999).

Dnmt1 is a complex protein harboring an N-terminal domain that suppresses *de novo* methylation and a C-terminal catalytic domain that is similar to bacterial methyltransferases (Bestor, 1992, 2000). The N-terminal domain also contains nuclear localization signals and a sequence that directs the enzyme to replication foci (Leonhardt *et al.*, 1992). In addition to Dnmt1s, the somatic form of Dnmt1 that is present in most cells, the *Dnmt1* gene

[1] To whom correspondence should be addressed. Fax: (215) 898-8780. E-mail: rschultz@sas.upenn.edu.

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produces two sex-specific transcripts (Mertineit *et al.*, 1998). Dnmt1p is found in pachytene spermatocytes; its 5′ exon interferes with translation, thereby preventing production of the protein during male germ cell meiosis. In contrast, very high levels of an N-terminal truncated active Dnmt1o protein are translated from the growing oocyte-specific transcript. The Dnmt1o protein persists until the blastocyst stage of development, when it is subsequently replaced by Dnmt1s (Mertineit *et al.*, 1998). Dnmt1o exhibits an unusual localization pattern during female germ cell and preimplantation mouse development (Carlson *et al.*, 1992; Mertineit *et al.*, 1998). In the growing oocyte, the protein is predominantly nuclear, but it is subsequently localized to the cytoplasm. Dnmt1o persists in the cytoplasm until the blastocyst stage with one exception: At the eight-cell stage, the protein is transiently localized to the nucleus. This cytoplasmic localization is surprising given the important role Dnmt1 is proposed to play in maintaining global methylation patterns. Its localization at replication forks in somatic cells aptly positions the enzyme to propagate this epigenetic modification to the newly replicated DNA. While evidence suggests that Dnmt1o is actively maintained in the cytoplasm (Cardoso and Leonhardt, 1999), little is currently known about how this localization is controlled.

We report here that Dnmt 1o is retained following permeabilization procedures that release more than 75% of total soluble protein. Moreover, the enzyme is still retained following treatments that disrupt either microfilaments or microtubules. Dnmt1o does not colocalize with either mitochondria or endoplasmic reticulum, but may colocalize with annexin V. In addition, the movement of Dnmt1o from the cytoplasm to the nucleus is independent of DNA replication, transcription, and protein synthesis, as well as compaction, cell contact, and cytokinesis. Thus, the movement into the nucleus appears to be a function of the time following fertilization.

MATERIALS AND METHODS

Oocyte and Embryo Collection and Culture

Oocytes were collected from PMSG-primed CF1 mice (Harlan) in Minimal Essential Medium supplemented with 3 mg/ml PVP and 0.2 mM IBMX as previously described (Schultz *et al.*, 1983). Two-cell, four-cell, and eight-cell embryos were flushed from the oviduct/uterus of PMSG and hCG-primed mice mated to B6D2F1 males (The Jackson Laboratory) as previously described (Manejwala *et al.*, 1986). Embryos were cultured in KSOM + amino acids (Erbach *et al.*, 1994; Ho *et al.*, 1995) in 5% CO_2:5% O_2:90% N_2 at 37°C. Embryos were cultured in medium containing either 3 μg/ml of aphidicolin or 100 μM fluorodeoxyuridine to inhibit DNA replication, 0.5 μg/ml cytochalasin D to inhibit cytokinesis, 10 μM nocodazole to inhibit microtubule polymerization, 24 μg/ml α-amanitin to inhibit transcription, or 10 μg/ml cycloheximide to inhibit protein synthesis. Further details are described in Results.

Isolation of Individual Eight-Cell Blastomeres

Uncompacted eight-cell embryos were isolated 67 h post-hCG. The *zona pellucida* was removed by a brief treatment with acid Tyrode solution, pH 2.5, and the embryos were then cultured in calcium-free KSOM for 30 min. Individual eight-cell blastomeres (1/8 blastomeres) were then isolated by gentle manipulation with a mouth-operated micropipet. Single 1/8 blastomeres were then cultured for 12 h in either calcium-containing or calcium-free KSOM.

Generation of Cytoskeletal Preparations

Cytoskeletal preparations of embryos were generated by placing the embryos in intracellular buffer (ICB; 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 20 mM Hepes, pH 6.8) containing 1% Triton X-100. After 10 min, the embryos were then washed through three drops of PBS containing 3 mg/ml PVP. These preparations were used for either confocal microscopy or Dnmt activity assay.

Immunocytochemical Detection of Dnmt1o and Annexin V

Embryo fixation, permeabilization, and laser-scanning confocal microscopy were performed as previously described (Doherty *et al.*, 2000); all images in each figure were taken at the same settings. The Dnmt1 antibody PATH52 was the generous gift of Dr. Timothy Bestor. Although this antibody recognizes both the Dnmt1o and Dnmt1s isoforms, Dnmt1s is not detected in the oocyte and preimplantation embryo, i.e., only Dnmt1o is expressed during this time (Howell *et al.*, 2001), and hence the observed staining is solely attributable to Dnmt1o. Saved images were cropped in Photoshop 3.0 and then assembled in Canvas 6.0. We have noted that there is a certain degree of variability from experiment to experiment in the appearance of the cytoplasmic punctate staining pattern and the intensity of the signal. The basis for this variability is not known.

To detect annexin V, oocytes or four-cell embryos were freshly collected from PMSG/hCG-primed CF1 mice then paraformaldehyde fixed for 1 h. The cells were next incubated for 1 h with 1:250 dilution of an antibody to annexin V (Santa Cruz Biotechnology; cat. no. sc-1928) and then examined by laser-scanning confocal microscopy. Specificity of staining was established by demonstrating that incubating the antibody with an excess of annexin V peptide (Santa Cruz Biotechnology; cat. no. sc-1928p) abolished detecting any signal (data not shown). Saved images were cropped in Photoshop 3.0 and then assembled in Canvas 6.0.

DNA Methyltransferase Assay

Total DNA methyltransferase activity was performed as previously described (Doherty *et al.*, 2000); ten embryos were used for each assay, which was conducted in triplicate.

Metabolic Radiolabeling of Embryos

Embryo proteins were metabolically radiolabeled by incubating embryos in KSOM containing 1 mCi/ml of [^{35}S]methionine (1500 Ci/mmole; Amersham) for 2 h in 5% CO_2:5% O_2:90% N_2 at 37°C. The total amount of acid-insoluble radioactivity material was determined as previously described (Poueymirou and Schultz, 1987).

Localization of Mitochondria and Endoplasmic Reticulum

Freshly collected eight-cell embryos were cultured for 1 h in KSOM supplemented with MitoTracker Green FM (200 nM final concentration) (Molecular Probes; cat. no. M-7514) or ER Tracker (200 nM final concentration) (Molecular Probes; cat. no. M-7514). The embryos were then fixed and processed for Dnmt1o detection by laser-scanning confocal microscopy as described above.

RESULTS

Cytoplasmic Retention of Dnmt1o

In somatic cells, Dnmt1s is localized in the nucleus at replication forks (Leonhardt *et al.*, 1992). Presumably, this promotes efficient methylation of the appropriate cytosine residues on the newly replicated strands and hence maintains this epigenetic modification. In stark contrast is the observation that the majority of Dnmt1o is localized in the cytoplasm of the oocyte and preimplantation embryo prior to the eight-cell stage. This observation is especially interesting in light of the finding that when the oocyte-specific form of Dnmt1 is expressed in somatic cells, it is located in the nucleus (Cardoso and Leonhardt, 1999). Taken together, these results suggest that the preimplantation embryo may be unique in its ability to retain Dnmt1o in the cytoplasm. Although the punctate staining patterning (Carlson *et al.*, 1992; Cardoso and Leonhardt, 1999; Doherty *et al.*, 2000) is consistent with a cytoplasmic retention mechanism, it is possible that Dnmt1o freely diffuses into the nucleus but then is rapidly and efficiently exported to the cytoplasm.

To ascertain whether Dnmt1o was indeed retained in the cytoplasm and not freely diffusible, four-cell embryos were treated with 1% Triton X-100, and the remaining insoluble material was then assayed for DNA methyltransferase activity. This treatment released the majority of soluble protein, since following permeabilization of metabolically radiolabeled embryos ~75% of the acid-insoluble radioactive material was released. Nevertheless, ~70% of the total amount of DNA methyltransferase activity in the embryos was retained following this treatment (Fig. 1). This residual activity was fairly tightly associated with these preparations, since it remained for at least 20 min following Triton X-100 treatment. It was unlikely that either microfilaments or microtubules were involved in the retention mechanism, since similar amounts of DNA methyltransferase activity remained associated with the Triton X-100-insoluble material when the embryos were initially treated for 2 h with either cytochalasin D (% retained, 79 ± 8) or nocodazole (% retained, 73 ± 4), which disrupt microfilaments and microtubules, respectively.

The presence of an insoluble DNA methyltransferase activity following Triton X-100 treatment was confirmed by laser-scanning confocal microscopy (Figs. 2A and 2A′). Dnmt1o was readily observed in the Triton X-100-treated embryos, and, if anything, the signal intensity was somewhat higher than the untreated controls. While the reason for this is not known, it could be attributed to greater access of the primary antibody as a result of the treatment. It should also be noted that Dnmt1o, which was present in the nuclei of the control nonpermeabilized eight-cell embryos, was absent from the nuclei of the permeabilized embryos. The absence of Dnmt1o in the nuclei of the permeabilized embryos confirmed that the nuclear envelope was indeed permeabilized, and moreover suggested that any association of Dnmt1o with nuclear components was weak. Last, consistent with the aforementioned result that neither microfilaments nor microtubules seemed involved in the cytoplasmic retention of DNA methyltransferase activity was the observation that similar amounts of Dnmt1o were retained in embryos treated with either cytochalasin D (or latrunculin A, which disrupts microfilaments by a different mechanism; Ayscough, 1998) or nocodazole prior to Triton X-100 treatment (Figs. 2B–2C′). Similar results were also observed when oocytes were treated with either cytochalasin D or nocodazole prior to Triton X-100 treatment (data not shown).

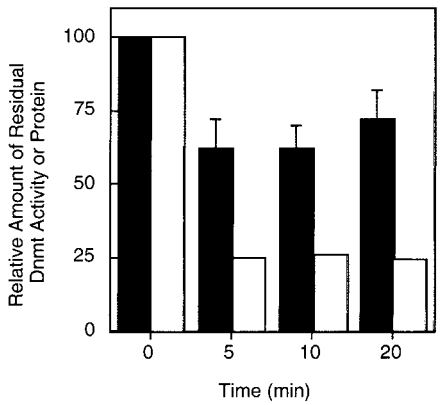

FIG. 1. Retention of Dnmt1o activity in Triton X-100-permeabilized embryos. Dnmt1o activity (solid bars) or residual amount of acid-insoluble radioactivity (open bars) in embryos treated with Triton X-100 for the indicated times. The experiment was conducted seven times. The data, which are expressed as the percent of untreated embryos, are the mean ± SEM. The errors are too small to be observed for the open bars.

The punctate staining pattern observed for Dnmt1o and its cytoplasmic retention could be due to its association with the mitochondria or endoplasmic reticulum. To determine whether such an association existed, four-cell embryos were stained with MitoTracker or ER Tracker, and Dnmt1o was then localized by immunostaining in these embryos. No apparent colocalization, however, was observed (Figs. 3A–3C and 3D–3F).

The amino terminus of Dnmt1o can bind annexin V (Ohsawa *et al.*, 1996), which is a calcium-sensitive phospholipid binding protein (Schlaepfer *et al.*, 1987). Such an interaction could account for the enhanced cortical Dnmt1o staining in oocytes and early cleavage-stage em-

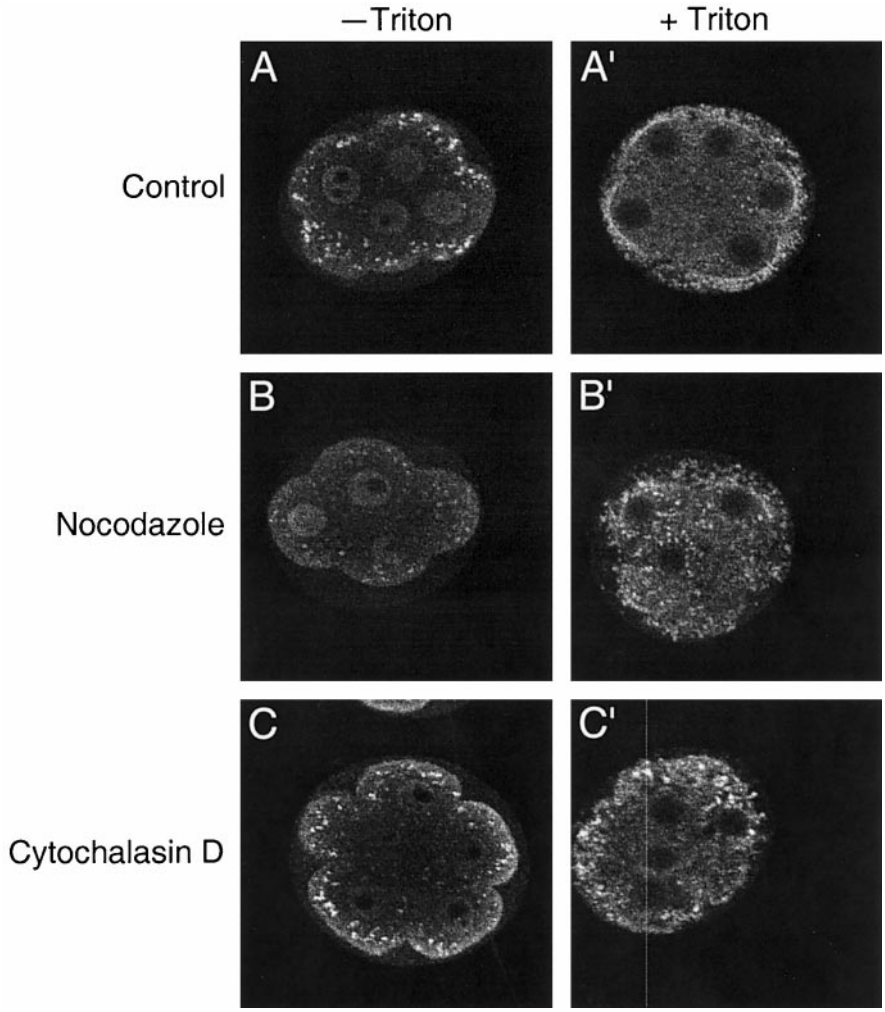

FIG. 2. Dnmt1o localization in permeabilized, nocodazole- and cytochalasin-treated embryos. Eight-cell embryos were incubated in medium containing either nocodazole or cytochalasin D for 2 h, permeabilized with Triton X-100, and then processed for immunocytochemical detection of Dnmt1o. (A–C) Unpermeabilized untreated control, nocodazole-, and cytochalasin D-treated embryos, respectively. (A′–C′) Permeabilized untreated control, nocodazole-, and cytochalasin D-treated embryos, respectively. The experiment was performed four times with at least five embryos in each treatment group. Shown are representative photomicrographs.

bryos and the retention of Dnmt1o following permeabilization. In fact, staining of either oocytes or four-cell embryos for annexin V revealed a discernible colocalization of Dnmt1o and annexin V (Figs. 3G–3I and 3J–3L). This colocalization was also observed in eight-cell embryos, and the annexin V present in the oocytes and embryos also remained associated with Triton X-100-insoluble preparations (data not shown).

Role of Cell Contact in Dnmt1o Nuclear Translocation

While Dnmt1o is retained in the cytoplasm during most of preimplantation development, it is translocated to the nucleus during the eight-cell stage, i.e., during compaction. This occurrence suggests a linkage between these two events. Prior to compaction, the individual blastomeres of the developing embryo are clearly discernible under the light microscope (Levy *et al.*, 1986). During compaction, the blastomeres flatten on one another such that following compaction it is difficult to delineate clearly the boundaries between the blastomeres. Compaction, which is mediated by the calcium-dependent cadherin E-cadherin (also called uvomorulin) (Hyafil *et al.*, 1980, 1981), is inhibited by incubating embryos in calcium-free medium. Accordingly, we examined the effect of inhibiting compaction on the nuclear translocation of Dnmt1o.

Four-cell embryos were cultured in calcium-free me-

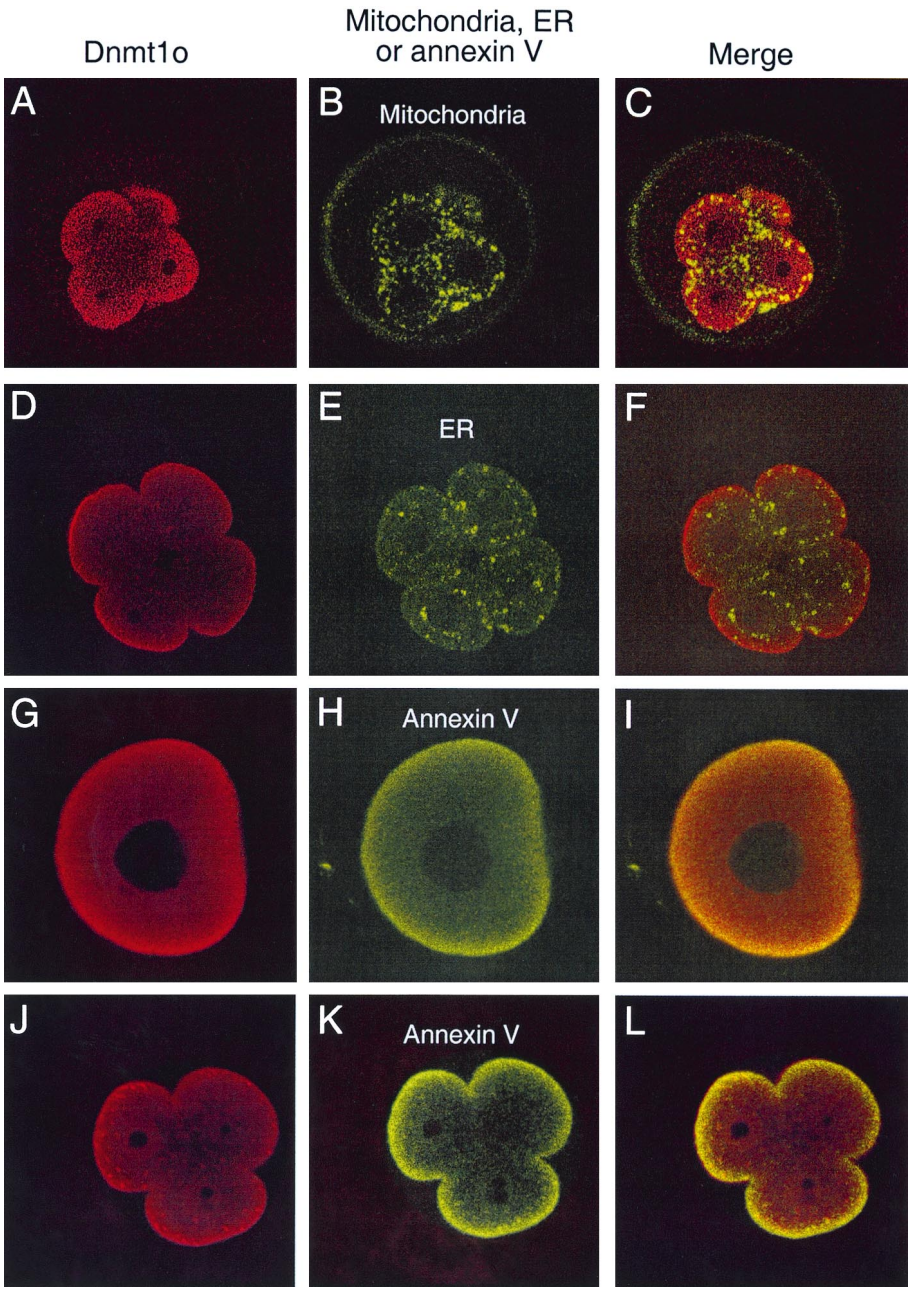

FIG. 3. Localization of Dnmt1o, mitochondria, endoplasmic reticulum, and annexin V. Oocytes or embryos were incubated with Mito Tracker or ER Tracker. The cells were then processed for Dnmt1o immunocytochemical localization. For annexin V, the cells were processed for simultaneous localization of both annexin V and Dnmt1o. (A–C) Localization of Dnmt1o and mitochondria in four-cell embryos. (D–F) Localization of Dnmt1o and endoplasmic reticulum in four-cell embryos. (G–I) Localization of Dnmt1o and annexin V in oocytes. (J–L) Localization of Dnmt1o and annexin V in four-cell embryos. The MitoTracker, ERTracker, and annexin V immunolocalization experiments were performed 4, 1, and 2 times, respectively, and at least 10 embryos were examined in each experiment. Shown are representative photomicrographs.

dium, and Dnmt1o was detected in these uncompacted embryos during and following the time that compaction occurred in the control embryos; there was no evidence of a nuclear localization of Dnmt1o in these four-cell embryos (data not shown). As previously reported (Carlson *et al.*, 1992; Doherty *et al.*, 2000), in control embryos cultured in

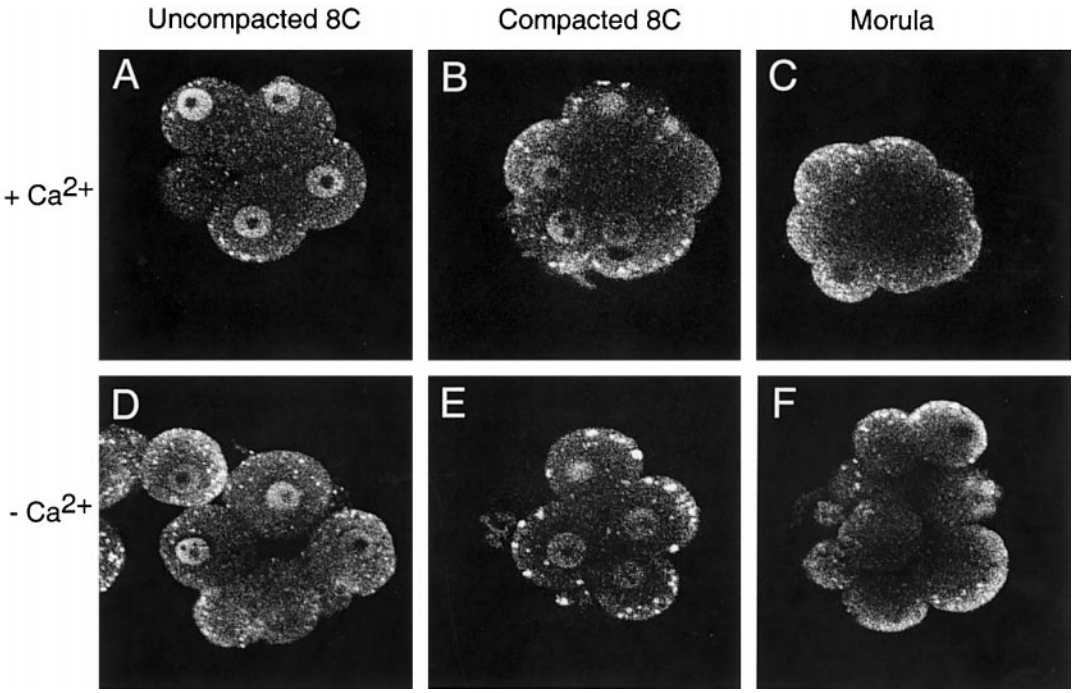

FIG. 4. Effect of inhibiting compaction on Dnmt1o nuclear localization. Four-cell embryos were cultured in either calcium-containing medium (A–C) or in calcium-free medium (D–F). Embryos were processed for Dnmt1o immunocytochemical localization when the embryos were uncompacted (A, D), compacted (B, E), or at the morula stage (C, F). Note in the compacted embryos the tight apposition of the blastomeres, in contrast to the loose blastomere apposition in the embryos cultured in calcium-free medium. The experiment was done three times, and for each experiment, approximately five embryos in each group were examined. Shown are representative photomicrographs.

calcium-containing medium, compaction was associated with the translocation of Dnmt1o into the nucleus, followed by exit of Dnmt1o by the morula stage. When the embryos were cultured in calcium-free medium, a similar nuclear translocation was observed during the eight-cell stage, followed by an exit by the morula stage (Fig. 4). Thus, the changes in cytoplasmic and nuclear localization of Dnmt1o appeared uncoupled to compaction.

When compaction is inhibited by culture in calcium-free medium, there still is a substantial amount of cell–cell contact between the blastomeres. To minimize this as a contributing factor, uncompacted eight-cell embryos were disaggregated into 1/8 blastomeres that were then cultured in isolation, i.e., in the absence of any cell contact. In these experiments, there was no sign of Dnmt1o nuclear localization in the donor uncompacted embryos (Fig. 5A). When the control embryos had undergone compaction and Dnmt1o was clearly localized in the nucleus, Dnmt1o localization in the cultured 1/8 blastomeres was assayed and also found to be nuclear (Fig. 5D). When the control embryos reached the morula stage, Dnmt1o was localized in the cytoplasm (Fig. 5C), as it was in the 1/8 blastomeres that had now cleaved to form two 1/16 blastomeres (Fig. 5E). This nuclear entry and exit of Dnmt1o also occurred when the 1/8 blastomeres were cultured in calcium-free medium (data not shown).

Compaction can be induced prematurely by treating four-cell embryos with biologically active phorbol esters or diacylglyerols (Bloom, 1989; Winkel *et al.*, 1990); the effect is transient and persists for a few hours. We found that treatment of four-cell embryos with TPA induced compaction within 30 min and that there was no evidence for a nuclear accumulation of Dnmt1o when the embryos were examined 0.25, 0.5, 1.0, 3.0, 4.0, and 5.5 h later (data not shown).

In toto, these results suggest that the temporal redistribution of Dnmt1o that occurs between the four-cell and morula stages is cell-autonomous and not linked with compaction and cell contact.

Role of Transcription and Protein Synthesis in Dnmt1o Nuclear Translocation

There are three periods of dramatic changes in gene expression that occur during preimplantation development. The first occurs during the maternal-to-embryonic transition during the two-cell stage, the second during the time of compaction at the eight-cell stage, and the third during the

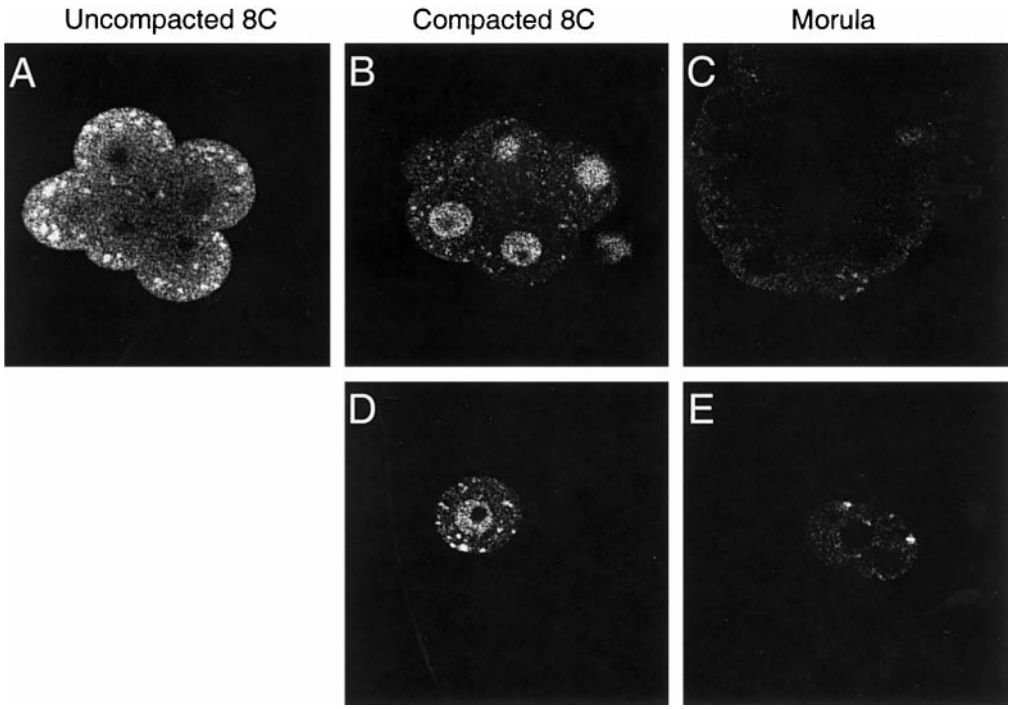

FIG. 5. Dnmt1o nuclear localization in 1/8 blastomeres. Single 1/8 blastomeres were obtained from uncompacted eight-cell embryos (A), as described under Materials and Methods, and then cultured to times when control embryos had either compacted (B) or reached the morula stage (C). Dnmt1o was localized in the nucleus of the 1/8 blastomere (D) that was chronologically at the compacted eight-cell stage and again resident in the cytoplasm at a time corresponding to the morula stage (E). The experiment was performed five times with at least five embryos in each treatment group. Shown are representative photomicrographs.

morula-to-blastocyst transition with the differentiation of the trophectoderm cells and establishment of the totipotent inner cell mass cells. In principle, the changes in gene expression that occur prior to or during the eight-cell stage could be involved in the nuclear translocation of Dnmt1o. This possibility was assessed by examining the effect of inhibiting transcription with α-amanitin or inhibiting the synthesis of any newly synthesized proteins from such expressed transcripts with cycloheximide.

Four-cell embryos were cultured in the presence of either inhibitor, and Dnmt1o localization was assessed when the embryos were chronologically at the eight-cell stage; inhibiting either transcription or protein synthesis prior to late G2 inhibits cleavage to the eight-cell stage. Results of these experiments indicated that Dnmt1o had translocated to the nucleus (Fig. 6), and hence the translocation event was independent of both transcription and protein synthesis.

Role of Cell Division in Dnmt1o Nuclear Translocation

To ascertain whether the nuclear translocation of Dnmt1o was linked to cell division, four-cell embryos were cultured in cytochalasin D. Cytochalasin D inhibits cytokinesis but not karyokinesis; the temporal sequence of the treated embryo's molecular development remains the same as that of untreated cells (Pratt *et al.*, 1981). Results of these experiments clearly demonstrated that Dnmt1o was present in the nuclei of the binucleate blastomeres that were chronologically at the eight-cell stage and absent in the nuclei of the binucleate blastomeres that were chronologically at the morula stage (Fig. 7). Thus, these intracellular movements are not linked to cytokinesis. In addition, we found no evidence for a premature movement of Dnmt1o into the nuclei of the cytochalasin D-treated embryos (data not shown). As discussed below, this suggests that the cytoplasmic retention of Dnmt1o was unlikely mediated by microfilaments that are known to be disrupted by the culture conditions employed (Pratt *et al.*, 1981).

The two major morphological transitions that occur during preimplantation development, namely, compaction and blastocoel formation, are independent of the proximal two rounds of DNA replication (Dean and Rossant, 1984; Manejwala *et al.*, 1986). To examine the role of DNA replication in the intercellular movements of Dnmt1o, two-cell embryos were cultured in medium containing aphidicolin, which inhibits replicative DNA polymerases (Ikegami *et al.*, 1978). If the two-cell embryos had completed S phase, they divided to and arrested at the four-cell

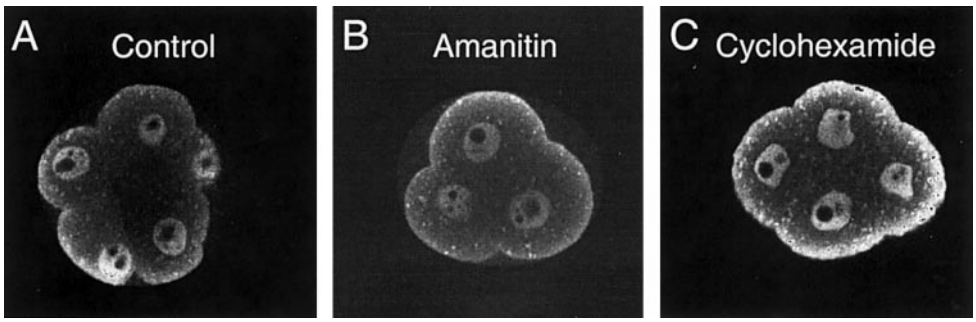

FIG. 6. Effect of inhibiting transcription and translation on Dnmt1o nuclear localization. Four-cell embryos were cultured in medium containing either α-amanitin or cycloheximide and then processed for immunocytochemical detection of Dnmt1o when the control embryos had reached the eight-cell stage and undergone compaction. (A) Control embryos, (B) embryos treated with α-amanitin, and (C) embryos treated with cycloheximide. The experiment was performed three times with at least three embryos in each treatment group. Shown are representative photomicrographs.

stage. If the two-cell embryos were in S phase, they remained arrested at the two-cell stage. Strikingly, Dnmt1o translocated into the nuclei of these aphidicolin-treated embryos at the same time that it was translocated in the control embryos, i.e., when the treated embryos were chronologically at the eight-cell stage (Fig. 8). Dnmt1o, how-

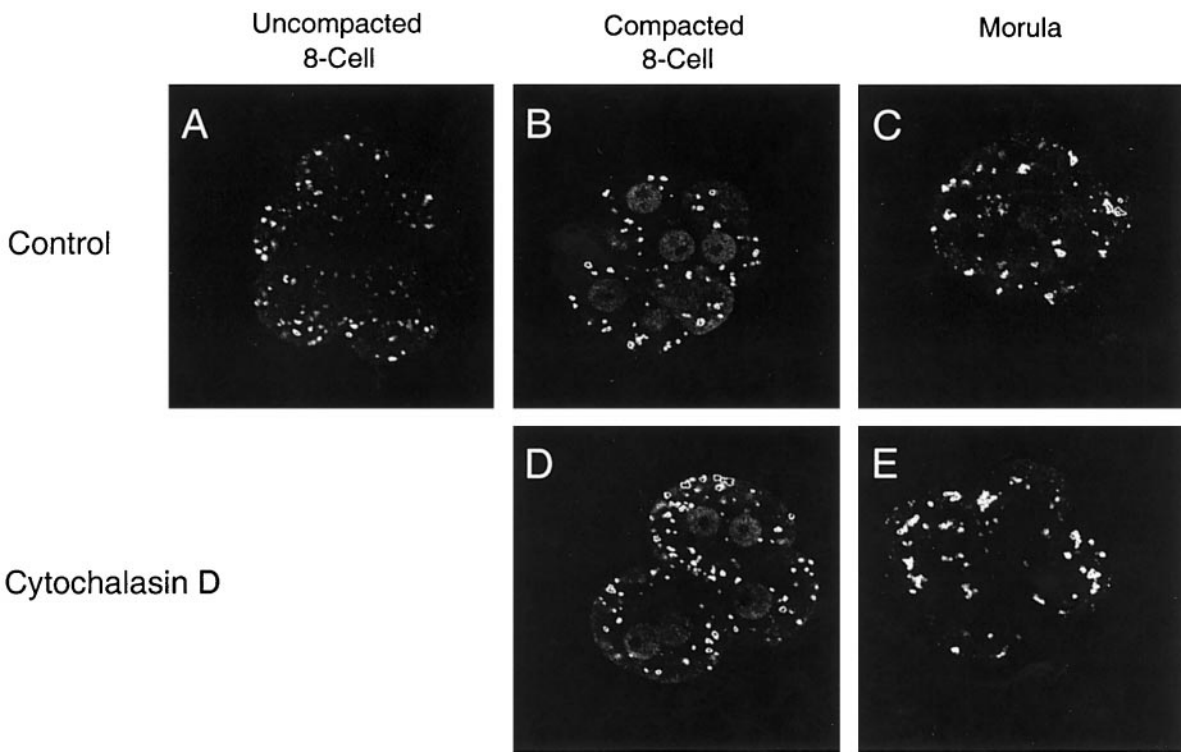

FIG. 7. Effect of inhibiting cytokinesis on Dnmt1o nuclear localization. Four-cell embryos were cultured in medium containing cytochalasin D and then processed for immunocytochemical detection of Dnmt1o when the control embryos had either compacted (B) or reached the morula stage (C); Dnmt1o was mainly cytoplasmic in the uncompacted eight-cell embryos (A). Dnmt1o was nuclear in the cleavage-arrested four-cell embryos that were chronologically at the compacted eight-cell stage (D) and cytoplasmic when they were chronologically at the morula stage (E). The experiment was performed two times with at least five embryos in each treatment group. Shown are representative photomicrographs.

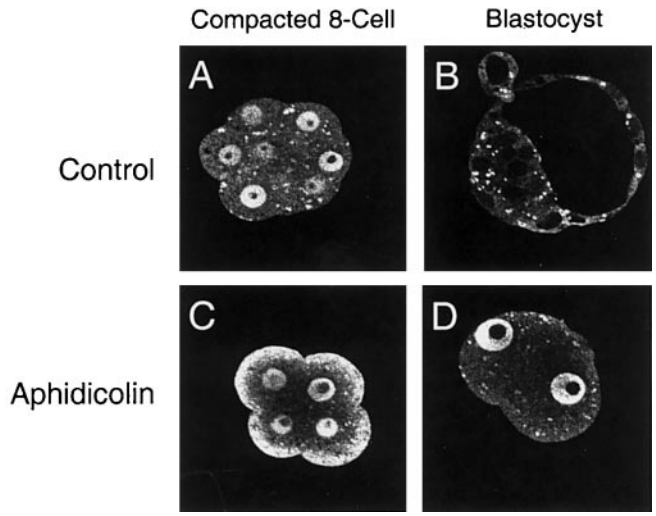

FIG. 8. Effect of inhibiting DNA replication on Dnmt1o nuclear localization. Two-cell embryos were cultured in medium containing aphidicolin and then processed for immunocytochemical detection of Dnmt1o when the control embryos had either compacted (A) or reached the blastocyst stage (B). Dnmt1o was nuclear in the cleavage-arrested two/four-cell embryos that were chronologically at the compacted eight-cell stage (C) and remained nuclear when they were chronologically at the blastocyst stage (D). The experiment was performed three times with at least four embryos in each treatment group. Shown are representative photomicrographs.

ever, remained localized in the nucleus following further culture and did not exit the nucleus, as was the case for the untreated embryos. Similar results were observed when DNA replication was inhibited with 5-fluorodeoxyuracil, which inhibits DNA replication by a different mechanism (Bolton *et al.*, 1984; and data not shown). Results of these experiments suggest that nuclear entry of Dnmt1o is a time-dependent event.

DISCUSSION

Several lines of experimentation indicate that differential DNA methylation is correlated with the expression of imprinted genes. For example, a region −4 to −2 kb upstream from the transcription start site of the paternal copy of the *H19* gene, which is not expressed, is hypermethylated relative to the maternal copy (Tremblay *et al.*, 1997), and deletion of this region on the paternal chromosome results in expression of the paternal allele (Thorvaldsen *et al.*, 1998). The ability of DNA-binding proteins, such as MeCP2, to recruit histone deacetylases could provide the mechanistic linkage between DNA methylation and repression of expression (Jones *et al.*, 1998; Nan *et al.*, 1998), since histone acetylation generates a transcriptionally permissive chromatin structure (Turner and O'Neill, 1995; Wolffe, 1996).

Following fertilization, a global demethylation initiates in the one-cell embryo and continues up to the blastocyst stage (Monk *et al.*, 1987; Sanford *et al.*, 1987), after which time *de novo* methylation occurs. The cytoplasmic location of Dnmt1o, despite the presence of a functional nuclear localization signal (NLS) (Cardoso and Leonhardt, 1999; Leonhardt *et al.*, 1992), may facilitate this global demethylation, since it would not be able to provide a maintenance function following each round of DNA replication. Remarkably, the allele-specific differences in DNA methylation of imprinted genes survive the global demethylation (Tremblay *et al.*, 1997). Thus, the transient nuclear translocation that occurs during the eight-cell stage (Carlson *et al.*, 1992; Doherty *et al.*, 2000) may perform a surveillance function to maintain the differential methylation pattern of imprinted genes, and consistent with this proposal is that deletion of maternal Dnmt1o results in loss of the paternal methylation pattern from one-half of the normally methylated paternal *H19* genes following fertilization (Howell *et al.*, 2001). At earlier times in preimplantation development, other DNA methyltransferase(s) may serve the maintenance function (Howell *et al.*, 2001).

While these observations suggest a role for the cytoplasmic retention of Dnmt1o and its transient nuclear localization at the eight-cell stage in genomic imprinting, little is known regarding the molecular basis for its cytoplasmic retention and what regulates the timing of Dnmt1o's nuclear entry. Results described here suggest Dnmt1o is retained in the cytoplasm, possibly by associating with annexin V, rather than being free to enter the nucleus and then actively exported, and that a molecular clock governs the time for nuclear entry.

Results from a previous study (Cardoso and Leonhardt, 1999) also suggest that Dnmt1o is retained in the cytoplasm by embryos despite the presence of an NLS, since a Dnmt1o–β-galactose fusion protein is localized in the nucleus when expressed in somatic cells. Moreover, incorporation of the SV40 NLS fails to direct a nuclear localization of Dnmt1o when it is expressed in preimplantation embryos. Last, overexpression of Dnmt1o–β-galactose fusion protein in preimplantation mouse embryos results in nuclear, as well as cytoplasmic, localization and suggests that cytoplasmic retention is saturable. Our results confirm and extend these observations by documenting that the bulk of both Dnmt1o immunoreactive material and enzyme activity is retained following permeabilization conditions that result in the release of the majority of oocyte/embryo protein, i.e., Dnmt1o is retained, and not free to diffuse, in the cytoplasmic compartment. Also consistent with this interpretation is that embryos treated with leptomycin B, which inhibits exportin 1/CRM1-mediated nuclear export (Kudo *et al.*, 1999), fail to evince any sign of nuclear accumulation of Dnmt1o over the period of 0.5–5.5 h (unpublished observations); nuclear accumulation should have been observed if Dnmt1o was free to enter the nucleus and then actively exported via the exportin 1/CRM1 pathway.

The molecular basis for the cytoplasmic retention, however, remains enigmatic. Disruption of the cytoskeleton by either microfilament or microtubule inhibitors does not result in the release of either Dnmt1o activity or immunoreactive material in the permeabilized embryos. Disruption of microtubules often leads to the collapse of the intermediate filament population around the nucleus, e.g., Franke *et al.,* 1978. The failure to observe any detectable redistribution of Dnmt1o localization in nocodazole-treated embryos minimizes an interaction with intermediate filaments. Although the punctate Dnmt1o staining that we and others (Cardoso and Leonhardt, 1999; Carlson *et al.,* 1992) observe suggests that Dnmt1o could associate with an organelle, we observe no apparent colocalization with either mitochondria or endoplasmic reticulum. Nevertheless, we do observe colocalization with annexin V, a protein known to interact with Dnmt1o (Ohsawa *et al.,* 1996). If a physical interaction exists, however, it still does not explain the cytoplasmic retention of Dnmt1o, since when Dnmt1o is expressed in somatic cells, which also express annexin V, it localizes to the nucleus (Cardoso and Leonhardt, 1999). It will be of particular interest to ascertain the localization of Dnmt1s, which contains an additional 118 amino acids at the amino terminus (Mertineit *et al.,* 1998), following expression in the oocyte/embryo. Retention of Dnmt1s in the cytoplasm would suggest that there is some unique quality of the oocyte/embryo cytoplasm that directs Dnmt1 retention (e.g., an oocyte/embryo-specific anchoring protein or posttranslational modification of Dnmt1), whereas nuclear entry would suggest that the amino-terminal sequence of Dnmt1s somehow prevents cytoplasmic retention.

The entry of Dnmt1o into the nucleus during the eight-cell stage is independent of DNA replication, transcription, translation, cell contact, and cytokinesis, since it occurs when each of these processes is inhibited or disrupted. Thus, the time of Dnmt1o's entry into the nucleus seems governed by a "molecular clock" that is uncoupled to these aforementioned processes. In fact, a molecular clock appears to govern each of the three major developmental transitions that occur during preimplantation development, i.e., genome activation (Schultz, 1993), compaction (Levy *et al.,* 1986), and blastocoel formation (Dean and Rossant, 1984; Pratt *et al.,* 1981), since each of these transitions appears uncoupled to cell cycle progression and initiates after a set period of time following fertilization. Although one could envision a coupling of nuclear translocation of Dnmt1o with compaction, such is not the case, since Dnmt1o enters the nuclei of eight-cell embryos in which compaction is prevented by either culture in calcium-free medium or in individual 1/8 blastomeres. Consistent with a dissociation of nuclear translocation from compaction is that inducing premature compaction does not induce the nuclear translocation. We also noted that, when protein synthesis is inhibited starting at the four-cell stage, there is no precocious entry of Dnmt1o prior to the eight-cell stage (unpublished observations). This result minimizes the likelihood that (1) the turnover of a protein involved in the cytoplasmic retention of Dnmt1o governs the time of nuclear entry of Dnmt1o, since it would be anticipated that such a putative protein would turn over faster in the absence of continual protein synthesis and (2) some protein(s) synthesized during the four-cell stage are required for nuclear translocation. Although inhibiting protein synthesis has no apparent effect on the time of nuclear translocation of Dnmt1o, inhibiting protein synthesis during the four-cell stage accelerates the time for onset of blastomere flattening (Levy *et al.,* 1986). This finding provides further evidence that, while the molecular pathways underlying compaction and nuclear translocation of Dnmt1o may share some components, they nevertheless are likely to require distinct components.

Interestingly, the only other protein to our knowledge that displays a transient nuclear localization during the eight-cell stage is PKC-ζ (Pauken and Capco, 2000), which is an atypical PKC in that it does not require either diacylglycerol or calcium for activation of protein kinase activity. Similar to Dnmt1o, inducing premature compaction by treatment with phorbol diesters does not induce a precocious PKC-ζ nuclear localization; it is not known whether this transient nuclear localization is also linked to a "molecular clock." In contrast to Dnmt1o, however, nuclear retention of PKC-ζ is observed in permeabilized eight-cell embryos (Pauken and Capco, 2000). Whether the nuclear translocation of Dnmt1o and PKC-ζ are mechanistically linked remains to be established.

Although Dnmt1o is again cytoplasmic following cleavage to the morula stage, it remains retained in the nucleus in aphidicolin-treated, cleavage-arrested embryos that are chronologically at the morula/blastocyst stage. While the failure to translocate to the cytoplasm may reflect impaired function of these embryos due to the prolonged inhibition of DNA synthesis, it may also implicate a role for cell division in the redistribution of Dnmt1o. Thus, nuclear envelope breakdown may be required for the release of Dnmt1o and its renewed cytoplasmic retention. Although we have never observed nuclear loss in late stage eight-cell embryos, it is possible that Dnmt1o is exported from the eight-cell nuclei. Analysis of the real-time kinetics of localization of a Dnmt1o–GFP fusion protein during the eight-cell to morula transition could resolve this question.

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